Gel-free proteomic methodologies to study reversible cysteine oxidation and irreversible protein carbonyl formation

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ABSTRACT

Oxidative modifications in proteins have been traditionally considered as hallmarks of damage by oxidative stress and aging. However, oxidants can generate a huge variety of reversible and irreversible modifications in amino acid side chains as well as in the protein backbones, and these post-translational modifications can contribute to the activation of signal transduction pathways, and also mediate the toxicity of oxidants. Among the reversible modifications, the most relevant ones are those arising from cysteine oxidation. Thus, formation of sulfenic acid or disulfide bonds is known to occur in many enzymes as part of their catalytic cycles, and it also participates in the activation of signaling cascades. Furthermore, these reversible modifications have been usually attributed with a protective role, since they may prevent the formation of irreversible damage by scavenging reactive oxygen species. Among irreversible modifications, protein carbonyl formation has been linked to damage and death, since it cannot be repaired and can lead to protein loss-of-function and to the formation of protein aggregates. This review is aimed at researchers interested on the biological consequences of oxidative stress, both at the level of signaling and toxicity. We are here providing a concise overview on current mass spectrometry-based methodologies to detect reversible cysteine oxidation and irreversible protein carbonyl formation in proteomes. We do not pretend to impose any of the different methodologies, but rather to provide an objective catwalk on published gel-free approaches to detect those two types of modifications, from a biologist's point of view.

Abbreviations

ROS: reactive oxygen species
RNS: reactive nitrogen species
Cys: cysteine
MS: mass spectrometry
TCA: trichloroacetic acid
IAM: iodoacetamide
MMTS: S-methyl methanethiosulfonate
DNPH: 2,4-dinitrophenylhydrazine
biotin-HPDP: N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide
LC: liquid chromatography
2-DE: two-dimensional gel electrophoresis
S-NO: S-nitrosylation
DTT :dithiothreitol
TCEP: tris(2-carboxyethyl)phosphine hydrochloride
ICAT: isotope-coded affinity tag
GELSILOX: gel-based stable isotope labeling of oxidized Cys
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
iTRAQ: isobaric tag for relative and absolute quantification
TMT: tandem mass tag
isoTOP-ABPP: isotopic tandem orthogonal proteolysis-activity-based protein profiling
NEM: N-ethylmaleimide
SNOSID: S-NO-Site Identification
SNO-RAC: S-NO Resin-Assisted Capture
SNOCAP: S-nitrosothiol Capture
ESNOQ: Endogenous S-NO quantification
SILAC: stable isotopic labeling by amino acids in cell culture
PUFA: polyunsaturated fatty acids
LPP: lipid peroxidation products
4-HNE: 4-hydroxyl-nonenal
ALEs: advanced lipoxidation end-products
AGEs: advanced glycation end products
GPR: Girard’s P reagent
BHZ: biotin hydrazide
ARP: aldehyde-reactive probe
O-ECAT: oxidation-dependent element coded affinity tag
HICAT: hydrazide-functionalized isotope coded affinity tag

Introduction: reversible and irreversible oxidative protein modifications

Free radicals and other highly reactive oxidants are continuously formed within cells due to aerobic metabolism. Under normal physiological conditions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are usually produced at physiological levels, in a regulated way and through different mechanisms [1]. Excessive production of ROS or impairment of cellular antioxidant defenses generates oxidative stress, which may promote oxidative modifications in all types of biomolecules, altering their function, and even leading to cell death.

Proteins are major targets of oxidants due to their high abundance in biological systems, and they can either undergo reversible modifications, which also can be part of their normal function and even be necessary for signaling, or suffer irreversible oxidative damage at various amino acids generally associated with loss of function [2-4]. The reaction of ROS and RNS with proteins leads mainly to three different kinds of oxidative protein modifications.

The first one is the oxidation of the side chains of amino acids, being the thiol group of cysteine (Cys) the primary target for oxidation reactions. Methionine and the aromatic amino acids tyrosine and tryptophan represent also sensitive targets of ROS and RNS though generally to a lesser extent than Cys. Oxidation of the side chains of the sulfur containing amino acids Cys and methionine leads to the formation of disulfides, sulfenic acid and methionine sulfoxides that correspond to reversible oxidative modifications. It should be mentioned, however, that under stronger oxidizing conditions, the already unstable sulfenic (-SOH) acid can be further oxidized to the irreversible oxidative forms sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acid, and methionine sulfoxide can be oxidized to sulfone [5] or even homocysteic acid [6].

A second type of oxidative modification is the oxidation of the carbon-nitrogen skeleton of the protein, leading to the fragmentation of polypeptide chains and/or the formation of intra or inter-molecular cross-links [7].

Thirdly, proteins can suffer carbonyl formation either by direct oxidation of sensitive amino acid side chains (lysine, arginine, proline, threonine) or due to the fragmentation of the carbon chain. Protein carbonyl formation can also take place by reacting with reactive carbonyl species that result from the

peroxidation of lipids or from the oxidation of reducing sugars (see section 4: 'Protein carbonyl formation and detection').

By virtue of the reversible nature of Cys or methionine oxidation, these modifications are non toxic and have been usually attributed with a dual role: protection from irreversible damage as ROS scavengers and modulation of protein function. Therefore, redox reversible protein modifications, particularly Cys thiol oxidation, are usually an early cellular response, if not the direct sensors, to mild oxidative stress. As such, thiol redox modifications play a very important role in redox signaling pathways [1,8-15].

After providing general premises to isolate and characterize oxidized proteomes (section 2: 'General pipeline to characterize an oxidized proteome'), this review will specifically summarize the state-of-the-art methodologies to study reversible Cys oxidation (section 3: 'Study of Cys oxidation at the proteome level by MS') and irreversible protein carbonyl formation (section 4: 'Protein carbonyl formation and detection') using mass spectrometry (MS). We have chosen these two types of modifications as representative examples of protein oxidation events linked to signaling or to toxicity, respectively.

General pipeline to characterize an oxidized proteome

The two main issues that have to be taken into account in redox proteomics are the labile nature of ROS-induced modifications that usually do not survive the purification steps needed to extract proteins from the biological sources, and the dynamic nature of these modifications that often affect only a small percentage of a given protein. Sample preparation, enrichment methods to facilitate the identification of modifications and the highly specific and sensitive analytical methods to detect these modifications are the major points to consider in a redox proteomics study. Fig. 1 outlines the general workflow for such a study.

Step 1. Preparation of protein extracts

The most important point to consider when preparing samples for analysis of protein oxidations is that the biological oxidation cannot be perturbed and artefactual oxidation events must be kept to a minimum.

To prevent perturbations of reversible Cys modifications during sample preparation it is necessary to block thiolate reactivity. As discussed below, this is easily accomplished by obtaining protein extracts in the presence of trichloroacetic acid (TCA). These acidic conditions below the pKa values of reactive thiolates allow their complete protonation, and they also stop thiol-disulfide exchanges by denaturing and precipitating proteins [16]. A different alternative, although kinetically less efficient, is to add membrane permeable Cys-alkylating agents, such as iodoacetamide (IAM) or N-ethylmaleimide (NEM), to the cellular media some minutes before cell lysis or to obtain the protein extracts in a buffer with the presence of saturating concentrations of Cys-reacting agent. Recently, the use of methylmethanethiosulfonate (MMTS) *in vivo* instead of IAM or NEM has been demonstrated to effectively block thiol oxidation during protein extraction [17]. In fact, the prevention of Cys reactivity with the use of Cys-alkylating agents is better recommended than acidic extraction for studying S-nitrosylations, since the generation of this modification is favored in acidic environments ([18] and references there-in).

The preservation of carbonyl modifications is not so easily accomplished as carbonyl groups in store samples readily undergo Schiff base formation with lysine residues of proteins, even when stored at -20 to -80°C [19] (our own results). This instability makes it necessary to both use fresh samples and limit the number of steps in sample preparation with all the carbonyl derivatization methods for isolating and identifying oxidized proteins. Some of the more important issues of sample preparation for protein carbonyl analysis have been recently reviewed [20]. For instance, it is important to use extraction buffers without reducing agents that can reduce carbonyls to alcohols [21]. In the particular case of carbonyls, it is also important to consider that other biomolecules (nucleic acids and carbohydrates) might become carbonylated and this can cause high background signal and increase sample complexity [22].

To avoid the presence of carbonylated nucleic acids, mild extraction strategies may be applied to minimize disruption of nuclei and mitochondria and leakage of nucleic acids by using hypotonic lysis buffers and avoiding strong detergents and sonication (19) and/or by precipitating nucleic acids with 1% streptomycin sulfate [23]. To avoid the presence of reduced carbohydrates with carbonyl groups, it is possible to clean protein extracts by selective removal of carbohydrates by lectin affinity or by the use of protein specific extraction methods like TCA precipitation following peptide-*N*-glycosidase F treatment [24].

Step 2. Specific labeling approaches of oxidized amino acids in extracts

Previous to the analysis of samples it is necessary to treat the protein extracts with reagents that are specific for the target modification (i.e. alkylating agents for thiols, dimedone for sulfenic acids, or hydrazides for carbonyls) (the specific manuscripts describing the use of each one of these reagents are provided below within each specific modification to be analyzed). This derivatization step may consist on just the addition of a chemical group that will facilitate the detection and analysis of the oxidative modifications, or may involve the binding of a tag that will allow the enrichment of samples, and therefore, reduce their complexity, or the quantification of these modifications if isotopic tags are used. Alternatively, protein extracts can be incubated with specific antibodies that recognize a specific modification (e.g. antibodies against some adducts of lipid peroxidation products with proteins) or that recognize the chemical group that has been previously added to the oxidized amino acid (e.g. antibodies against the 2,4-dinitrophenylhydrazine (DNPH) generated by the derivatization of carbonyls with hydrazides). In any case, it is important to perform the derivatization reactions in conditions that allow the maximal efficiency and specificity. This is sometimes in conflict with the conditions required to obtain maximal solubility of proteins. This problem has been specifically addressed by Fedorova and colleagues for the derivatization of carbonyls in proteins [25].

Step 3. Enrichment

Although this is an optional step, the purification of samples by pull-down or immuno-precipitation allows the easier identification of oxidized modifications that often exist in sub-stoichiometric levels and that, additionally, can occur in low abundant proteins. However, the enrichment step has some drawbacks that need to be considered, particularly if the enrichment strategy is based on biotin labeling. Biotinylated labeling and enrichment is widely used, but one concern, as with all enrichment based strategies, is the specificity of the enrichment. In biotin-based capture methods there may be a carryover of endogenously biotinylated proteins or non biotinylated proteins that are complexed with oxidized and biotin-labeled proteins. This limitation is overcome when reversible biotin labeling is used [e.g. N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP)] and recovery is performed by reduction of the disulfide

linker. Finally, another caveat is the influence of the tag on the retention time in liquid chromatography (LC) and the fragmentation in MS. For instance, the derivatization of carbonyls with hydrazides generates tags that are relatively large and hydrophobic. This increases the retention times in reverse-phase HPLC and, therefore, reduces the separation efficiency. On the other hand, it has been shown that biotin-hydrazide-tagged peptides may not be successfully identified from their tandem mass spectra [26]. The reasons are probably the steric interference of the tag during fragmentation, as well as the relatively crowded spectra due to neutral losses and secondary fragmentations of the tag [27].

Step 4. Sample mixing

As outlined in Fig. 1, mixing of samples is performed if they have been labeled with isotopic or isobaric tags to either compare protein abundance or the extent of protein oxidation, depending on the functionality of the tag, between two different experimental or genetic conditions. This step needs to be done at the earliest stage possible to avoid the quantification bias that could arise from the analytical steps. The rationale behind isotopic labeling is that stable isotopes are used to differentially introduce a predictable mass difference between peptides deriving from different samples. After labeling, samples can be either digested into peptides and mixed in 1:1 ratio, or mixed before proteolytic digestion, and analyzed by LC-MS/MS. The mass shifts caused by the different tags will yield multiple peaks in MS spectra, which are used to relatively quantify proteins in the original samples and will also indicate the modified amino acid residue. In the particular case of isobaric tags, labeled proteins or peptides have identical masses and chemical properties. It is only after peptide fragmentation by collision-induced dissociation (CID) during MS/MS that reporter ions have different masses. This is achieved thanks to the structure of isobaric tags consisting of three components: a mass reporter that has a unique number of ^{13}C substitutions, a mass normalizer that has a unique mass that balances the mass of the tag to make all of the tags equal in mass, and a reactive moiety that reacts with proteins through primary amines, Cys or carbonyls.

Step 5. Sample detection by MS – limitations of two-dimensional gel electrophoresis approaches

The most widely used analytical techniques in redox proteomics experiments are two-dimensional gel electrophoresis (2-DE) and MS. However, the intrinsic limitations of 2-DE and the developments in the last decade of high performance mass spectrometers have prompted a switch from gel-based to gel-free, MS-based proteomics [28]. In 2-DE approaches, proteomes are separated by means of electrophoresis and modified proteins are detected by Western blotting or with specific fluorescent labeled probes. The main disadvantages of 2-DE are that protein separation by electrophoresis is labor-intensive, it is poorly reproducible and, because of its limited dynamic range of sensitivity and limited protein load on gels, associated with sub-stoichiometric modifications in one single spot, the identification of less abundant and low oxidized proteins becomes very difficult. In addition, the incomplete recovery of peptides from a gel digest may worsen the sensitivity. Finally, and not less important, more than one protein can occupy the same position in 2-DE gels, and after positive identification by MS of several proteins in only one spot it may be impossible to confirm which one of them was really oxidized. The scope of this review is to describe gel-free redox proteomic strategies; some recent reviews have specifically addressed gel-based redox proteomic methodologies as well as some recent advances in the use of redox fluorescent probes [29-31].

Study of Cys oxidation at the proteome level by MS

In 2001, Jaffrey and collaborators devised a very clever strategy to study S-nitrosylation (S-NO) in proteins, taking advantage of the highly reactive nature of the thiolate anion [32]. This strategy was called the biotin-switch method and consisted in the following (Figure 2, right red panel). First, all free and reduced thiols in a complex protein mixture are blocked with MMTS, then ascorbate is used to selectively reduce all S-NO to thiols, and finally, the newly formed thiols are tagged with a biotin moiety through a mixed disulfide bond after reacting them with biotin-HPDP. This last step would allow either the direct detection of S-nitrosylated proteins after immuno-blotting with antibodies to biotin, or protein enrichment after affinity purification of the S-nitrosylated proteins with immobilized avidin and then release by reduction of the disulfide linker followed by identification of S-nitrosylated proteins by MS analysis.

This strategy was key for the development of new methodologies for the detection and quantification not only of protein S-NO but also all other types of reversible thiol modifications, including

sulfenic acids, protein S-glutathionylation or all reversible Cys oxidations in a non-selective manner. The basic requirement to adapt the methodology for the study of the desired modification is to change the type of reducing agent to be used after the first alkylating step. Thus, as above mentioned, S-NO modifications are detected by using as a reductant ascorbate [32], or ascorbate in combination with $CuCl_2$ [33], which provides improved sensitivity without loss of selectivity; sulfenic acid modifications can be identified upon reduction with arsenite [34,35]; the *Escherichia coli* Grx3 C14S/C65Y double mutant was reported to specifically reduce S-glutathionylated Cys [36,37], and the general reducing agents dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) non-selectively reduce all types of reversible Cys modifications [38-40]. Some of these techniques will be described in the following paragraphs together with other innovative ways to approach the study of thiol redoxomes.

Study of reversibly oxidized thiol proteomes

Several reports of reversible oxidized thiol proteomes started to appear soon after the pioneering biotin switch assay of Jaffrey and colleagues [32]. Thus, the groups of Jakob and Toledano developed similar strategies as the biotin-switch assay but initially using TCA for freezing the thiol redox status to characterize the reversibly oxidized thiol proteomes of *E. coli* and *Saccharomyces cerevisiae,* respectively [41,42]. However, these studies relied on the use of 2-DE with its own intrinsic limitations, including lack of sensitivity, which favors the detection of those proteins with higher expression levels in a protein extract, and lack of selectivity, as by our own experience, several different proteins are detected when a single spot is analyzed by MS.

The adaptation of the gel-free quantitative isotope-coded affinity tag (ICAT) technology to redox proteomics was a step forward in the characterization of reversible oxidized thiol proteomes. The ICAT reagents were initially designed for quantifying changes in protein levels between two compared samples [43]. They consist in a thiol-reactive IAM moiety linked to a biotin tag through a cleavable 9-carbon linker, which exists either in an isotopically light ([^{12}C] ICAT) or a 9-Da-heavier isotopically heavy ([^{13}C] ICAT) form. Taking advantage of the thiol-reactive side of the ICAT reagents, Sethuraman and colleagues [44,45] initially used the ICAT reagents to identify and quantify protein thiols in a rabbit heart membrane which undergo oxidation upon a high concentration of H_2O_2 compared to a control untreated sample. So,

they used the ICAT reagents to label the reduced thiols in both the control untreated (light ICAT) sample and the treated (heavy ICAT) sample. After ICAT labeling, the samples were mixed, trypsinized, and affinity purified prior to LC-MS/MS. The ICAT reagents are chemically identical, so they co-elute from the HPLC system and equally behave on the mass spectrometer. Since they are 9 Da different in mass, 9 Da differences or multiples of 9 Da reflect the number of oxidized Cys residues in a peptide, and the relative peak areas of the MS of light and heavy labeled peptides allow their relative quantification. Thus, they were able to quantify the increase of oxidation (reversible and irreversible) as a decrease in signal for a given pair of light and heavy ICAT labeled peptides belonging to the untreated and treated samples, respectively. This technique is not only quantitative but also it allows the identification of the involved redox-sensitive Cys by MS/MS sequence analysis of the light and heavy labeled peptides, and is amenable for the identification and quantification of thousand of peptides at the same time.

It was four years later when the group of Jakob reported the study of the thiol redox proteome in *E. coli* after oxidative treatment with H_2O_2 and sodium hypochlorite stress utilizing the ICAT reagents but with a different perspective [46]. In this case the ICAT reagents were used to label both reduced and oxidized thiols within the same biological sample, and the technique was called OxICAT. Thus, in the first alkylation step all reduced thiols are labeled with light ICAT, then all reversible oxidized Cys are reduced with TCEP and newly forming thiols are labeled with heavy ICAT. The sample is then trypsinized and all ICAT labeled peptides are enriched in an affinity purification step by using the biotin tag of the ICAT moieties. The biotin tag is cleaved and samples are processed by LC-MS for quantification of the redox modification and by tandem MS/MS for identification of the peptide sequence and the site of Cys oxidation. In this technique the relative peak areas of the same peptide labeled with light and heavy ICAT reagents reflect its absolute oxidation status, independent of the variations of the protein concentration. This same workflow was successfully followed in the identification and quantification by the same group of reversible Cys oxidations in *E. coli* [47], *S. cerevisiae* [48], *Caenorhabditis elegans* [49,50] and lysates and sub-cellular fractions of mammalian cells [51,52] under different environmental and genetic conditions. Even though this technique is powerful, it harbors some practical constraints. Probably, the main one is the high difference between the ranges of concentration between oxidized and reduced Cys, as most of the intracellular Cys residues are in a reduced state compared to those oxidized (5 to 10%)

[53]. This means that in order to be able to find ICAT pairs for quantification extensive sample fractionation is required, making data analysis complex and time-consuming. Moreover, the amount of sample to be used for a single experiment is limited to around 100 µg, as recommended by the manufacturer, and requires the use of two ICAT reagents per analyzed sample. As for other methods using ICAT reagents (see below), it is important to point out the importance of checking the blocking efficiency of the ICAT reagents, since they are normally used at significantly lower concentrations that when cheap blocking reagents are used instead.

We and others envisioned a different type of strategy for studying the reversible thiol proteome using the ICAT reagents [13,54-57]. Our proposed workflow consists in the comparison of two samples, control untreated and oxidized treated (environmental or genetic condition), at once. Thus, for the two samples to be compared, reduced thiols are blocked with IAM, then reversible oxidized thiols are reduced with TCEP and newly appearing reduced thiols (formerly oxidized) are labeled in one of the samples with the light ICAT reagent and in the other one with the heavy ICAT reagent. After labeling, both samples are mixed together and trypsinized, and the ICAT labeled peptides are then enriched by affinity purification followed by biotin tag cleavage and LC-MS/MS for peptide quantification and sequence analysis. By following this approach, the level of complexity of the sample to be analyzed is reduced due to a higher similitude between the relative abundances of peptides containing oxidized Cys. Also, it is possible to increase the amount of the sample by 20-fold relative to the OxICAT approach at the same time that there is a reduction in the number of ICAT reagents to be used in a pairwise comparison, which reduces expenses. A minor limitation of this approach is that it requires an additional step of protein quantification to distinguish between changes in Cys oxidation versus protein concentration. However, this can be easily achieved by quantifying the proteins of the non-enriched fractions belonging to the same samples by either label-free or stable isotope-based MS protein quantification. Thus, by combining ICAT labeling with N-terminal dimethyl labeling [58], we were able to quantify and identify reversible oxidized thiols in *S. pombe* upon oxidative stress [54] and in different genetic backgrounds [55].

Other strategies using solid platforms have been recently developed for the analysis of the reversible oxidized Cys proteome. GELSILOX (gel-based stable isotope labeling of oxidized Cys) is one of them [59]. This consists on first alkylate all reduced thiols in protein extracts prior to dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE), which is run a short distance until they form a single band containing the whole proteome. This part of the gel is then used as a reaction chamber for reduction of reversible oxidized Cys, alkylation of the newly formed thiols with a different Cys reagent and protein trypsinization. After digestion peptides belonging to two different samples are eluted from the gel and labeled with either ^{16}O or ^{18}O prior to MS. With this approach both the relative abundances of oxidized versus reduced Cys and the protein levels are quantified simultaneously in a single experiment by using a computational approach that analyzes variance at the peptide level, and which allows the quantification of small changes in the Cys redox status under subtle oxidative stress conditions. This methodology was used to detect Cys-oxidative changes in different endothelial cell lines subjected to oxidative treatments and to evaluate the oxidative damaging effects of ischemia-reperfusion in subsarcolemmal mitochondria isolated from rat hearts cardiomyocytes which were subjected to IR ischemia-reperfusion. Another approach which is becoming popular is the use of a resin capture by thiol-disulfide exchange chemistry. The workflow is similar to the biotin switch assay, first reduced thiols are alkylated, those reversibly oxidized are then reduced with a general reducing agent (DTT or TCEP) and newly formed thiols are then captured by a thiol-reactive resin (thiopropyl Sepharose). Proteins can then be trypsinized on the resin and labeled with different isotopic reagents including the 4-plex isobaric tags for relative and absolute quantification (iTRAQ) or the 6-plex tandem mass tags (TMT) for quantitative LC-MS/MS analysis [60,61].

Other approaches were developed in order to identify functional Cys on a proteomic scale. Thus isotopic tandem orthogonal proteolysis-activity-based protein profiling (isoTOP-ABPP) allows the quantitative analysis of native Cys reactivity [62]. This approach uses an IAM-based probe consisting of an alkyne functional group which allows performing ‘click chemistry’ conjugation to an azide-functionalized TEV-protease recognition peptide coupled to a biotin moiety for affinity enrichment of labeled peptides and a valine which exists either in the isotopically light or heavy form for quantitative MS. The approach consists in the comparison of the Cys reactivity within a proteome using two different concentrations of the light and heavy IAM-based probes. It is expected that differences in the extent of alkylation would reflect differences in Cys reactivity, so that hyper-reactive Cys would be completely labeled at low probe concentrations, whereas hypo-reactive Cys would show a concentration-dependent

increase in probe incorporation. This methodology was used to ascertain Cys reactivity profiles in several human cancer cell lines and mouse tissue proteomes, concluding that reactive Cys are enriched in functional residues, are rather conserved among eukaryotes and correspond to preferential sites for posttranslational modifications. This same approach was later used to identify the Cys more susceptible to H_2O_2 oxidation in two pathogenic bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* [63].

Study of sulfenic acid modifications

Cys sulfenic acids are difficult to detect due to the highly chemical instability of this species. Thus sulfenic acids not only are products of thiol oxidation but also are intermediates in the same oxidative process, and in the presence of proximal thiolates or amino groups, they become further oxidized to protein disulfides or sulfenamides, respectively. However, they also have an interesting chemical property, as they can act either as electrophiles or nucleophiles, depending on the surrounding environment, and this property has been used for the development of probes for their detection within either proteins or proteomes.

The selective direct and irreversible reaction of sulfenic acids with dimedone (5,5-dimethyl-1,3-cyclohexanedione) has been largely exploited for the detection of this oxidative modification in proteins (Fig. 2, left blue panel). Thus, the group of Poole modified a dimedone analogue, 1,3-cyclohexadione, with two different fluorophores, isatoic acid and 7-methoxycoumarin to 1,3-cyclohexadione, which allowed sulfenic acid detection in proteins, as confirmed by testing the reagents against a mutant sulfenic acid stable form of the peroxiredoxin AhpC from *Salmonella typhimurium* [64]. Later, other similar probes were designed and synthesized incorporating either other fluorescent groups (fluorescein and rhodamine) or biotin to the dimedone analogue [65] or to dimedone [66], the biotin tag allowing enrichment of the labeled proteins or peptides by affinity purification, the efficiency of the latter was tested in rat ventricular myocytes after different treatments with H_2O_2. To reduce the volume of the dimedone-biotin molecules, a new less bulkier probe specific for sulfenic acid detection was designed, the N-(3-azidopropyl) -3,5-dioxocyclohexanecarboxamide (DAz-1), allowing the direct detection of sulfenic acid modifications in living mammalian cells [67]. In this probe, the nucleophile for the specific reaction with sulfenic acids is the 1,3-cyclohexanedione, but it also contains an azide group, which permits the incorporation of other

moieties for detection or affinity purification by Staudinger ligation or 'click chemistry' after the labeling step. The direct reaction of dimedone with sulfenic acids also inspired the development of sulfenic-acid specific antibodies [68]. Thus a hapten-protein conjugated was generated by attaching dimedone to a cysteamine backbone with a carbon linker to keyhole limpet hemocyanin. Rabbit α-hapten-Ig gamma (IgG) antibodies were elicited with the resulting product and tested to image sulfenic acid in biological samples by immune-fluorescence staining and fluorescence microscopy and to detect sulfenic acid modifications on protein microarrays in lysates from cancerous human tissues and mammalian cell lines. After these initial studies using dimedone or derivatives as the backbone for the synthesis of sulfenic acid detection probes, other sulfenic acid reactive moieties were designed in order to improve ease derivatization and yield, reactivity at physiological pH, no intracellular ROS-induction and accumulation, and even reversibility, as compared with dimedone-based probes [69-72].

While all the methods above described allow detection and/or enrichment of sulfenic acids in proteins, none of them provides quantitative data regarding the extension of the modification in a particular condition. With this purpose, the group of Carroll reported two different methods based on isotope-coded reporters to detect and quantify relative levels of sulfenic acid modifications in protein extracts. In the first method, termed ICDID due to the use of isotope-coded dimedone ([D_6]-dimedone, heavy tag) and iododimedone (light tag) as probes [68], sulfenic acids are derivatized with [D_6]-dimedone, and free thiols are labeled with iododimedone. After protein digestion peptides are separated and analyzed by LC-MS, and relative values of sulfenic acid modification, are obtained by dividing the intensity of the heavy-isotope labeled peak of the sum of intensities of the heavy and light-labeled peaks. In the second method heavy 4-(3-azidopropyl)cyclohexane-1,3-dione ([D_6]-DAz-2) and light DAz-2 were used as probes [73]. In this case the probes allowed the conjugation of an acid-cleavable biotinylated tag via click chemistry after the labeling step, which permits protein/peptide enrichment by affinity purification. The labeling scheme for this method starts with the covalent modification of sulfenic acids in two protein samples to be compared with either light or heavy DAz-2, then sample mixing and conjugation of the probe with the cleavable biotin tag followed by affinity purification, release of the biotin tag with trifluoroacetic acid followed by peptide analysis and quantification by MS/MS. The principle of this methodology was demonstrated with a C64S C82S double mutant of yeast recombinant glutathione

peroxidase Gpx3, which is able to be oxidized to a sulfenic acid after H_2O_2 treatment in its catalytic Cys36.

A new recent publication reported a highly sensitive method for the relative or absolute quantification of sulfenic acid levels in peptides or in proteins, but without taking into account the site of modification [74]. This is based on the labeling of sulfenic acids and thiols with metal-containing probes, lanthanide-1,4,7,10-tetraazacyclododecande-1,4,7,10-tetraacetic acid-dimedone (Ln-DOTA-dimedone) and lanthanide-containing metal-coded affinity tag (Ln-MeCAT), respectively, and then use inductively coupled plasma MS, a highly sensitive technique, which moreover has a wide linear range and multiplexing capabilities, for highly specific detection and quantification. A standard peptide and β-lactoglobulin treated with different H_2O_2 concentrations were used to demonstrate the applicability of the above described procedure.

Study of disulfide bonds

Accomplishing the specific study of protein disulfides in a protein extract is complicated due to the intrinsic nature of this species that makes the development of specific-targeted probes and reducing agents extremely difficult, unless in the case of mixed disulfides with glutathione (S-glutathionylation, see below). Therefore, this modification is generally reported in combination with all other Cys reversible oxidations when adaptations to the biotin-switch assay are performed in the presence of general reducing agents such as DTT or TCEP.

However, two reports in 2004 [75,76] were able to adapt diagonal SDS-PAGE [77] to redox proteomics and reported some proteins undergoing disulfide formation after different oxidative stress treatments in a mammalian neuronal cell line and adult cardiac myocytes (Fig. 2, left blue panel). In diagonal SDS-PAGE oxidized proteins are separated in a first dimension by non-reducing electrophoresis. Then, a lane containing the separated proteins is excised from the gel and placed horizontally in a second gel to perform the second dimension by reducing electrophoresis. Therefore, the majority of the proteins will run in a diagonal except those forming disulfide bonds. Most of the proteins forming intermolecular disulfide bonds will run below the diagonal in the second dimension (because they migrate slower in the first dimension), and the opposite, proteins forming intra-molecular disulfide bonds

will migrate faster in the first dimension and are expected to run above the diagonal in the second dimension. However, the conformational changes suffered by many proteins forming intra-molecular disulfide bonds after being reduced under denaturing conditions can affect their expected migration pattern, thus limiting the usefulness of this approach. Since proteins are separated as spots in a gel, they can be excised and analyzed by MS. While being an interesting technique which may accomplish some of the goals of a proteomic-wide study of protein disulfide bonds, its main disadvantage is to deal with the intrinsic limitations of 2D electrophoresis (see above).

In the case of S-glutathionylation, the use of the *E. coli* Grx3 C14S/C65Y mutant as a reducing agent has facilitated the study of this Cys oxidative modification by indirect methods. Hence, a proteomics quantitative approach was recently reported for the identification and quantification of this Cys oxidative modification in RAW 264.7 mouse macrophages upon treatment with different diamide and H_2O_2 concentrations [37]. In this approach free thiols and selectively reduced S-NOs were initially alkylated with NEM. Then S-glutathionylated Cys were reduced by the *E. coli* Grx3 C14S/C65Y enzyme in combination with GSH and reduced cofactor NADPH, so that the newly formed thiols were later captured by a thiopropyl Sepharose 6B resin, which was used as a platform for digestion, and further amino-terminal labeling with iTRAQ reagents prior to LC-MS/MS for site-specific identification and quantification.

Study of S-nitrosylation

S-NO-Site Identification (SNOSID) was published in 2006 as the first high-throughput proteomic-wide gel-free extension of the biotin switch methodology and was applied to investigate S-NO modification in proteins from rat cerebellum lysates treated with different concentrations of S-nitrosoglutathione and in human aortic smooth muscle cells upon exposure to S-nitrosocysteine (CysNO) and propylamine propylamine NONOate (PAPANO) [78,79]. So, the first three steps of this technique are essentially the same as in the above described biotin-switch assay. However, the improvement of the SNOSID is the addition of a trypsinization step before affinity purification. In this way, peptides (formerly S-NO modified) instead of proteins are enriched by the avidin resin in the affinity purification step. These peptides are then resolved and analyzed by nLC-MS/MS and database searching, which results in the simultaneous

identification of both the sites of the modification in the protein and the protein to which the peptide belongs. A similar strategy named S-NO Resin-Assisted Capture (SNO-RAC) was later developed for studying this same type of reversible Cys modification in both human embryonic kidney cells and *E. coli* treated with CysNO S-nitrosocysteine and in RaW264.7 macrophages stimulated with cytokines [80]. In this case, a thiol-reactive resin is used instead of a thiol-reactive biotin, which allows labeling of the new formed thiols after S-NO reduction and affinity purification to be performed in a single step.

However, neither SNOSID nor SNO-RAC provided quantitative data regarding the extension of the S-NO modification on a proteome. An adaptation of the ICAT technique for the study of S-NOs is the S-nitrosothiol Capture (SNOCAP) which was used to assess the stability of protein S-NOs in cell lysates *in vitro* and in murine endothelioma cells [81]. In this procedure the biotin moiety used to selectively target S-NOs was synthesized in two different isotopic forms, 'heavy' and 'light', both comprising three main blocks: the disulfide required for reacting with thiols (former S-NOs), a linker containing heavy (^{13}C and ^{15}N) or light (^{12}C and ^{14}N) isotopes of carbon and nitrogen, respectively, and the biotin moiety required for peptide enrichment on a neutravidin resin. The advantage of having two different isotopic forms of the same molecule is the possibility of using them to compare two samples at once. Thus, in each sample, free thiols are alkylated, then S-NOs reduced with ascorbate/Cu^{2+}, and the new free thiols in each sample are differentially labeled with either the light or heavy forms of the biotin moiety. At this point both samples are combined for being trypsinized, enriched and further processed for quantification and identification analysis by LC-MS/MS. A similar quantitative approach is the Endogenous S-NO quantification (ESNOQ) methodology [82]. This is a combination of the biotin-switch technique with the stable isotopic labeling by amino acids in cell culture (SILAC) and LC-MS/MS. This technique provides quantitative data and was used for comparison of the extension of the S-NO modification in two different samples, lipopolysaccharide and interferon-γ-treated RAW264.7 cells and untreated control cells. The use of SILAC allows treatment and control cells to be mixed and processed together along all experimental steps, minimizing errors, however an important limitation relies on the sample type limitation that can be used with the SILAC methodology. Finally, another novel proteomic-based approach allows identifying and quantifying up to six different samples at once by using the Cys thiol-reactive isobaric tandem mass tag (CysTMT) sixplex reagents [83]. Each of the six CysTMT reagents has the same

nominal parent mass, react with thiols through their dithiopyridine group and incorporate a MS-neutral spacer arm and a MS/MS reporter, which are cleaved during MS/MS acquisition for ion fragmentation and sequence information yielding reporter ions with unique m/z ranging from 126 to 131 Da. The workflow is basically the same as for the biotin switch assay, first reduced thiols in the samples are blocked with NEM, then S-NO modified thiols in Cys are reduced with ascorbate, and the newly formed thiols are labeled with the CysTMT reagents. Samples are then mixed, trypsinized, and S-NO containing peptides are enriched with TMT affinity resin prior of being eluted under acidic conditions, which preserves the mass tag on the modified Cys. S-NO enriched and eluted peptides are subjected to LC-MS/MS and then identified by the site of CysTMT labeling and quantified by calculating the ratios of the CysTMT sixplex reporter ions. This approach was used to quantify S-NO in human pulmonary arterial endothelial cells *in vitro* and *in vivo* upon oxidation with different concentrations of S-nitrosoglutathione and using reduced and oxidized GSH as treatment controls. A slight modification of the reagents led to the development of the iodoacetyl tandem mass tags (iodoTMT) sixplex reagents, which also specifically react with thiols through their iodoacetamide group and give the same tag as the CysTMT reagents. These were also used to detect S-NO modifications in lipopolysaccharide-stimulated murine BV-2 microglial cells treated or not with S-allyl Cys [84].

Doulias and colleagues reported a new MS-based methodology in which S-NOs were identified and quantified after their direct reaction with phenylmercury in a solid phase to assess the in vivo S-NO proteome of the mouse liver [85] (Fig. 2, left blue panel). In this reported method, phenylmercury is conjugated to agarose beads (solid phase) or to a biotin molecule (liquid) providing a mechanism for 'capturing' proteins containing S-NO modifications. Mercury from phenylmercury forms a relatively stable thiol-mercury bond with the sulfur atom from the S-nitrosocysteine displacing the nitroso group from the sulfur atom. Since phenylmercury also reacts with sulfurs from free thiols, the first step is to block all reduced Cys in extracts with MMTS. Then, protein extracts are allowed to react with phenylmercury on the resin or biotinylated phenylmercury. Reacting proteins can be released from the resin by reduction of the mercury-sulfur bond with β-mercaptoethanol for downstream LC-MS/MS analysis. For identification of the modification site, proteins bound to the phenylmercury resin are in-column digested and released by mild oxidation with performic acid, which at the same time oxidizes Cys to sulfonic acid providing a +48

Da mass shift in the MS. With this methodology, the reducing step is bypassed therefore avoiding artifacts due to unspecific reductions as might be the case in all other biotin-switch based methodologies.

Organophosphines are another group of moieties that can react in a fast way with S-NOs yielding stable and detectable products (Fig. 2, left blue panel); particularly, triaryl-substituted phosphines are highly reactive towards S-NO. The substituent of the aryl ring determines the reaction mechanism and the final product [86-89], which if with enough stability can be further analyzed by MS [90]. However, no phosphine-based probe has been yet developed that allows peptide/protein enrichment, a fact that remains as a major challenge in the development of these types of approaches.

Protein carbonyl formation

Protein carbonyl formation is the irreversible introduction of carbonyl groups, such as ketones, aldehydes and lactams, on the polypeptide chain. It is considered the major hallmark of oxidative stress since the largest family of oxidized amino acid products is represented by carbonylated derivatives. Carbonyl groups may be introduced within the protein structure at different sites and by different mechanisms [91].

The first mechanism involves metal-catalyzed oxidation through the Fenton reaction [92]. The reduction of metal ions in the presence of H_2O_2 generates highly reactive hydroxyl radicals [93] that either oxidize amino acid side chains or cleave the protein backbone [7]. The oxidative cleavage of the protein backbone can occur by either the α-amidation pathway or by oxidation of glutamyl side chains [94]. On the other hand, the direct oxidation of proline and arginine side chains results in glutamic semialdehyde, whereas, lysine oxidation yields aminoadipic semialdehyde and threonine 2-amino-3-ketobutyric acid [95]. Besides these metal-catalyzed oxidation amino acid oxidations, direct oxidation of tryptophan by ROS generates at least seven oxidation products. Among them, kynurenine and N-formyl kynurenine and their hydroxylated analogues, carry an aldehyde or keto group formed by oxidative cleavage of the tryptophan indole ring, and oxindolylalanine and dioxindolylalanine carry a lactam group [96-98].

ROS can also oxidize polyunsaturated fatty acids (PUFA) from cellular membranes [99-102] generating lipid peroxidation products (LPP). These compounds have a longer half life than ROS and can diffuse into the cell, amplifying the damage of free radicals [99], 4-hydroxyl-nonenal (4-HNE), acrolein, malondialdehyde among others, are strong electrophiles and, as such, react with nucleophile

amine, thiol or imidazole groups, forming adducts with lysine, Cys and histidine and leading to the formation of the so-called advanced lipoxidation end-products (ALEs) [103,104]. The introduction of carbonyls by LPP on polypeptide chains can occur by either forming Schiff bases or by Michael type addition [105,106]. Michael adducts of α,β-unsaturated alkenals retain the carbonyl moiety of the LPP within the protein structure. Since LPP can attack the proteins by two different mechanisms, one single molecule can react with residues belonging to the same protein or two different proteins and cause intra or intermolecular cross-linking [107]. Two recent reviews address the formation of adducts between proteins and LPPs as well as the proteomic strategies to characterize them [108,109].

Protein carbonyl formation can also occur via glycooxidation. Glycation (i.e., the reaction of reducing sugars such as glucose or fructose with the side chains of lysine and arginine residues) forms Amadori and/or Haynes products that can be oxidized by ROS yielding advanced glycation end products (AGE) [91,110,111] that contain carbonylated moieties. Reactive α-carbonyls formed during glycooxidation, such as glyoxal, methylglioxal or 3-deoxyglucosone, modify the basic residues lysine and arginine to yield, for example, pyrralines and imidazolones [112,113]. The complex chemistry involved in the formation of AGE has been recently reviewed [114] as well as the proteomic strategies to analyze them [115].

The proteome-wide analysis of protein carbonyl formation using MS is still a particularly challenging task for several reasons: (i) the huge diversity of carbonylated species, as explained above, which under specific conditions, may occur simultaneously; (ii) carbonylated species are sub-stoichiometric, may be transient and may occur in already very low abundant proteins; since the non-modified isoform of the protein is usually present in higher amounts, MS would preferentially identify it; (iii) successful identification of protein carbonyl formation requires relatively stable modifications that are not lost during fragmentation of the peptides during the MS/MS procedure; (iv) high resolution MS instrumentation is required as some carbonyl modifications result in small mass shifts, such as aminoadipic semialdehyde formed from lysine (1 m/z unit), which are difficult to distinguish by fast-scanning mass analyzers with low resolutions. This problem can be, however, overcome by last generation high-resolution mass spectrometers such as Q-Exactive hybrid quadrupole-Orbitrap or Orbitrap Fusion. In addition, some oxidations of amino acids are isomeric, such as the oxidation of

proline that results in glutamic semialdehyde (a carbonyl oxidation) and hydroxyproline (a non carbonyl oxidation).

These limitations can be partially overcome by using different strategies to trap carbonyl-containing proteins prior to MS. In the next section we will briefly describe the most common chemistries/reagents (Fig. 3) and pipelines (Fig. 4) used for the enrichment of carbonyls in proteins. Many of these approaches have recently been reviewed in great detail [20,25].

Types of reagents used for the detection of protein carbonyls by MS

Although the sub-stoichiometric nature of oxidative modifications calls for labeling and enrichment of carbonys in proteins, strict label free approaches, that is, direct identification of carbonylated peptides without derivatization neither enrichment, have indeed been used to study protein carbonyl formation, particularly for 4-HNE-modified peptides as they display a reporter ion very easy to identify [116]. One advantage of label-free methods is that there is no protein loss due to protein precipitation when removing excess labeling reagent [20] and that, provided that samples are carefully prepared, label-free methods are usually more reproducible.

Most reports, however, describe the use of reagents able to first label and then enrich protein carbonyls based on two types of chemistries (Fig. 3). The first one is the reaction of the nucleophilic amine of hydrazides with carbonyl groups yielding hydrazones that can be further stabilized by reductive amination with sodium borohydride ($NaCNBH_3$) to yield an irreversible secondary amine bond (Fig. 3a). The second one is based on the reaction of a nucleophilic amine from an aminooxy group with carbonyl groups to result in the formation of oxime bonds, more stable than hydrazones (Fig. 3b). We will summarize in the next two sections many hidrazide- or aminooxy-based reagents used for the detection of protein carbonyl formation.

A third strategy to capture carbonyls, which has only been reported once in the literature, is not based on carbonyl reactivity, but on the click-chemistry between an azide and a terminal alkyne to yield a 1,2,3-triazole [117]. This strategy, however, has only been reported once in a study of proteins from colorectal cancer cells treated with 4-HNE. In this study, cells were treated with alkynil modified 4-HNE and protein carbonyls carrying an alkynil-4-HNE were incubated with a reagent with azido and biotin

groups separated by a photocleavable linker [118]. Proteins modified in this way were affinity-purified on streptavidin beads, released from the beads by photolysis with a low-intensity UV light, and identified by nLC-ESI-MS/MS [119].

Analysis of protein carbonyls in single biological samples by MS – use of non-isotopic/non-isobaric reagents

In most cases, reagents able to react with carbonyls following the two types of chemistries described above are used to irreversibly label *in vitro* and enrich protein carbonyls generated *in vivo,* and are retained in the peptides for MS analysis. In few examples, however, a hydrozide-based reagent immobilized on solid phases has been used to enrich carbonylated peptides, and unmodified peptides were then released from the solid matrix by reversing the hydrazone bond. After elution, peptides are then tag-free when analyzed by MS. Following this strategy, carbonyls in peptides were captured by a solid-phase hydrazide reagent [120] or even by immobilized oxalyldihydrazide on a microchip [121]. Hydrazide-functionalized resins allow the enrichment of peptides with carbonyls by covalent chromatography using reversible hydrazone formation as an efficient capture-release strategy. The enriched peptides can then be eluted by raising the temperature until 60°C and lowering pH with 10% trifluoroacetic acid or formic acid. Two recent reports have improved this tag-free methodology by labeling the amino groups of all peptides with isotopic reagents, prior to solid phase enrichment of peptides with carbonyls, to facilitate protein quantification of different biological samples (Fig. 4, blue panel): in the first report, Rauniyar and Prokai labeled parallel, non-enriched peptide samples by reductive methylation of the amino groups with light or heavy formaldehyde, before enrichment with hydrazide glass beads; it was possible to relatively quantify protein abundances from a digested matrix of human plasma in combination with the identification of modified peptides [122]. Similarly, the Maier's group published a similar approach, in which they derivatized peptides from different biological matrices, including rat heart mitochondrial proteomes, with isotope-coded-succinic anhydride that labels primary amines in peptides, and enriched them with commercially available hydrazine-functionalized agarose beads to detect, identify and quantify site-specific carbonyl modification [123].

Several reagents have been developed for irreversible labeling and enriching carbonylated proteins or peptides (Fig. 4, red panel, left). Among them, 2,4-dinitrophenylhydrazine (DNPH) was the first one to be used for the detection of carbonyls; it reacts with carbonyls forming the stable 2,4-dinitrophenylhydrazone. DNPH derivatization has since then been adapted to be used in the isolation, identification, and quantification of proteins with carbonyls [23] through selection of derivatized proteins with DNPH antibodies [124,125]. DNPH was originally introduced as reagent and reactive matrix in MALDI-TOF peptide mapping experiments for the characterization of carbonyls in protein species [126-128]. However, it has to be taken into consideration that DNPH and hydrazide labeling may be reversed during the proteolysis step in solution and in the diluted acid conditions typically used for nLC-ESI-MS/MS analysis. It has been shown that this reversibility depends on time and pH [129]. For that reason, derivatization with DNPH and all other probes based on hydrazide chemistry is usually followed by a reductive step stabilizing hydrazones with $NaCNBH_3$ [130-134]. One drawback related to the use of DNPH and hydrazides as a labeling strategy is that they cross react with sulfenic acids [135,136]. In any case, the use of reducing agents prior to labeling to reduce sulfenic acids should circumvent this cross-reactivity.

Derivatization with Girard's P reagent (GPR) [1-(2-hydrazino-2-oxoethyl) pyridiniumchloride] is also based on the reactivity of hydrazides with carbonyls but provides another route for the selection of peptides with carbonyls. Besides containing a hydrazide, GPR also contains a quaternary amine that allows quaternary amine containing peptides to be selected from mixtures by strong cation exchange resin at pH 6.0 [137]. As explained above for the use of DNPH, derivatization with GPR has to be followed by a reduction step with $NaCNBH_3$ to yield the stable derivatives. As opposed to other hydrazide-based tags, the advantage of this approach is that excess of derivatizing reagent does not have to be removed before chromatography analysis. In addition, derivatization with quaternary amines enhances peptide ionization through quaternization. Finally, this reagent enhances the solubility of proteins as oxidized proteins may have reduced solubility due to cross-linking, denaturation, and backbone cleavage. This reagent has successfully been applied to characterize carbonylated proteins in yeast [137]. A newly described reagent, Girard Reagent T (GRT), similar to GRP, has been applied to the study of carbonylation sites in monoclonal antibodies [138]. The advantages of GTR compared to

GRP, are that GRT has a higher solubility in water, which allows higher reagent-to-protein molar ratio for derivatization (given the same protein concentration), and a smaller molecular weight, which potentially provides slightly better site accessibility.

One of the most widely used probes to identify protein carbonyls and that has been validated in complex proteomic samples is biotin hydrazide (BHZ). There are, however, several other biotinylated probes that use the hydrazide chemistry, although not all of them are commercially available. In a study directed to evaluate the performance of these biotinylated probes, the authors concluded that none of the labeling groups were better in terms of spectrum counts, but some probes, e.g. PEG4-linked biotin hydrazide, were more efficient in reacting with protein carbonyls [139]. As with all hydrazides, carbonyl groups are first derivatized with BHZ to form a Schiff base and then the Schiff base is reduced to the more stable amines using $NaCNBH_3$ to prevent reversal of derivatization [140,141]. A further step to remove excess BHZ before avidin or streptavidin affinity chromatography, before enrichment, is necessary in this case to prevent the excess of biotin to saturate the beads. This is mostly accomplished by either dialysis or precipitation with TCA.

Aldehyde-reactive probe (ARP) is an O-(biotinylcarbazoylmethyl) hydroxylamine and, as such, it can react with carbonyl groups to generate the corresponding aldoxime/ketoxime derivatives. The advantages of ARP are the higher efficiency in labeling of both aldehyde and ketone-containing peptides compared to DNPH and BHZ, when used in acidic conditions, and that it does not require the stabilizing reduction step after carbonyl labeling [142]. However, as for BHZ, when ARP is used to affinity capture proteins/peptides with carbonyls further steps to completely remove the ARP excess, such as buffer exchange using centrifugal filters or TCA precipitation and washing [143,144] are necessary. ARP carbonyl derivatization has been used in complex samples such as human monocytic THP-1 cells treated with 4-HNE [143,144] or rat cardiac mitochondria [145].

All the described methodologies above can be complemented with other steps that will enable the measurement of the relative abundance of an oxidized protein, in addition to the identification of proteins/peptides with carbonyls. An example is the experiment that Madian and colleagues performed by labeling with different N-terminal-labeling iTRAQ isomers labeled with biotin [146].

Simultaneous analysis of protein carbonyls in multiple biological samples by MS – use of isotopic/isobaric reagents

Isotopic/isobaric labeling of carbonyls enables both simultaneous, qualitative and quantitative characterization of modified peptides thanks to the carbonyl enrichment and isotope-based quantification within one single labeling reaction (Fig. 4, red panel, right). Some of the reagents used for isotopic labeling are isotopically-labeled versions of those normally used for carbonyl derivatization. This is the case for ^{13}C6-labeled-DNPH or deuterated GRP. However, some other tags have been specifically designed with the purpose of carbonyl site identification and quantification.

O-ECAT (oxidation-dependent element coded affinity tags) is a carbonyl-specific reagent [(S)-2-(4-(2-aminooxy)-acetamido)-benzyl)-1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid] that contains an aminooxy group, able to covalently bind aldehyde or ketone end products, and a metal chelating moiety that can be complexed with different rare earth metals to generate specific mass tags [147,148]. After derivatization of carbonyl groups with O-ECAT, the chelating moiety is used to bind rare earth metal such as Tb (158.9 Da) or Ho (164.9 Da). Treating different protein samples with different rare earth metals allows differential coding of samples. After coding, mixing and tryptic digestion, labeled peptides are enriched using an antibody against the metal chelating moiety of O-ECAT and analyzed by MS. This strategy has only been tested in oxidized proteins [147,148] and requires validation in complex biological samples.

HICAT is a hydrazide-functionalized isotope-coded affinity tag that presents three functional parts [149]. The first one is a hydrazide group reacting with ketones/aldehydes. The second one is a linker composed by four C atoms that can be either ^{13}C in the heavy form or ^{12}C in the light form, and that, therefore differ by 4 Da. This part is necessary for the relative quantification. The third part is a biotin moiety for peptide enrichment by avidin affinity chromatography. After tryptic digestion of the different samples, peptides from different samples are differentially coded and mixed. Labeled peptides are enriched and further fractioned before analysis by MS/MS analysis. This strategy was used for the identification and quantification of *in vivo* oxidized proteins from rat cardiac mitochondria [149].

Some isobaric labels have been recently synthesized, but they have not been yet been applied to proteome-wide analysis or even to protein carbonyl analysis. The first of them is the iTRAQH reagent

which is identical to iTRAQ but with hydrazide functionality. It was recently generated by simple, one step conversion, of amine-reactive N-hydroxysuccinimide ester to hydrazide moiety in the presence of excess hydrazine [150]. In addition to the advantages of isotopic labeling reagents, the isobaric nature of the tags allows multiplex analysis of up to 8 samples, increasing throughput analysis and quantitative precision. However, one important limitation is the lack of a linked chemical group for the specific enrichment of peptides with carbonyls. So far, iTRAQH reagents have only been used with purified proteins, but not in complex biological samples, and to the best of our knowledge they are not commercially available.

An alternative to iTRAQH are the aminooxy-TMT or carbonyl-reactive TMT reagents. This hybrid tag exists with two different functionalities, hydrazide or aminooxy. It contains a functional moiety for carbonyl labeling and a TMT moiety for quantification and enrichment. By using this carbonyl-reactive TMT label, proteins with carbonyls could be directly quantified from up to 6 biological samples [151]. An additional advantage is that labeled proteins/peptides may be immune-purified and/or immune-detected using anti-TMT antibody. These reagents have so far only been used in the field of glycomics [151], but not yet applied to protein carbonyl analysis.

Final conclusions

In this review, we have summarized the different MS-based strategies for the detection, identification and quantification of reversible and irreversible protein oxidations, namely Cys oxidation and carbonyl formation in amino acid side chains. Although most of the strategies have indeed been validated in complex proteomes, we also show some new reagents that have only been used in purified proteins, as indicated above within each specific section. We do not pretend to impose any specific methodology, but, rather, show the different possibilities that a biologist may use to respond to his/her particular interest. In general, both for Cys oxidation and protein carbonyls, methods can be divided into two groups. The first group of methods allows the identification of oxidized amino acids in a single proteome and, in order to quantify oxidation levels, samples need to be analyzed in parallel. The second group use either the direct isotopic/isobaric labeling of the oxidized sites or the labeling of amino-termini of different samples, and allow the quantification of oxidized sites in two (isotopic labels) or up to eight (isobaric labels) samples

simultaneously. The final decision on which methodology to use will also depend on budget and available technical MS equipment.

Our personal choice for the study of reversible thiol oxidation in proteomes is the use of ICAT reagents, which allow the identification and quantification of all types of reversible Cys oxidation with standardized methodologies [54,57]. The method allows the analysis of two biological samples, and it is based on the 'freezing' *in vivo* of the thiol stage of Cys residues by medium acidification, followed by derivatization of reduced thiols in extracts with alkylating agents and sequential reduction and labeling of reversible oxidized thiols with the two commercially available ICAT reagents, so that the two samples can be compared at once by LC-MS/MS analysis after avidin-beads peptide enrichment. A further advantage is the elimination of the biotin tag allowing the easier identification of peptides by MS. An alternative to this approach, which would allow the comparison of up to six different biological samples at once, would be the use of the isobaric CysTMT [83] or iodoTMT [84] tags described above for the characterization of nitrosothiols. We do not know, however, whether the affinity resins proposed to enrich TMT-labeled peptides are as robust as the purification of biotin-labeled peptides by streptavidin beads.

Regarding quantification of protein carbonyls in whole proteomes by MS, we would suggest the use of the HICAT reagents, which are hydrazide-functionalized derivatives of the two ICAT isotopic reagents [149]. These reagents, not commercially available, were synthesized by the Maier's group, and their biotin tags are not cleavable, which may difficult the analysis by MS. It would be interesting to further develop these probes by adding cleavable linkers between the isotopic carbons and the biotin tag as for the ICAT reagents. Similarly useful probes seem to be the aminooxy-TMT reagents that would allow the quantification of carbonyls in amino acids for up to six samples [151]. As for iodoTMT, the proposed workflow would be the labeling of carbonyls in samples, mixing, digestion, enrichment through TMT affinity, and MS analysis. Despite their promising utility, more studies are definitely necessary to validate the use of iodoTMT or aminooxy-TMT for thiol or carbonyl, respectively, labeling, particularly regarding the use of anti-TMT antibodies for peptide enrichment, in terms of specificity and contamination with non labeled peptides.

Declaration of interest

There are no conflicts of interests. The authors alone are responsible for the content and writing of the paper.

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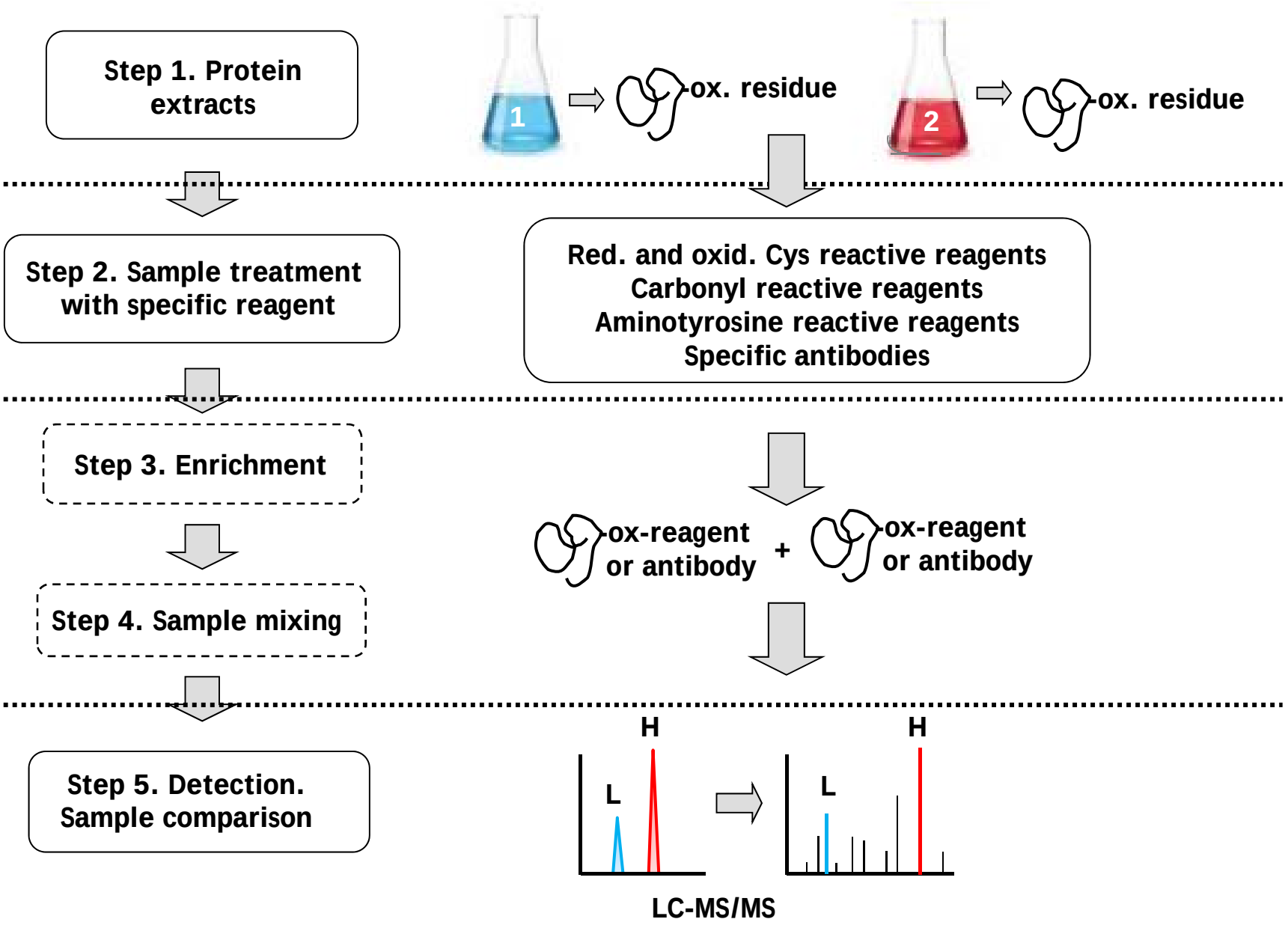
Figure Legends

Figure 1. General workflow for proteomic analyses of oxidative modifications in proteins. After protein extraction (Step 1), proteins are treated with reagents that will specifically label the oxidative modification (Step 2) of interest and that will enable the enrichment of oxidized proteins from complex proteomes (Step 3) and/or the identification of oxidized peptides by 2-DE or LC-MS/MS. Alternatively, proteins can be incubated with antibodies that may directly recognize the oxidative modification or the labeled modification. Once proteins are labeled and optionally enriched, they are subjected to LC-MS/MS (Step 5), to detect oxidized proteins and identify the modified amino acids. To compare the relative abundance of an oxidized peptide between two different samples, labeled samples with specific isotopic tags can optionally be mixed (Step 4) prior to detection and analysis. Optional steps in this workflow (Steps 3 and 4) are shown in dashed squares.

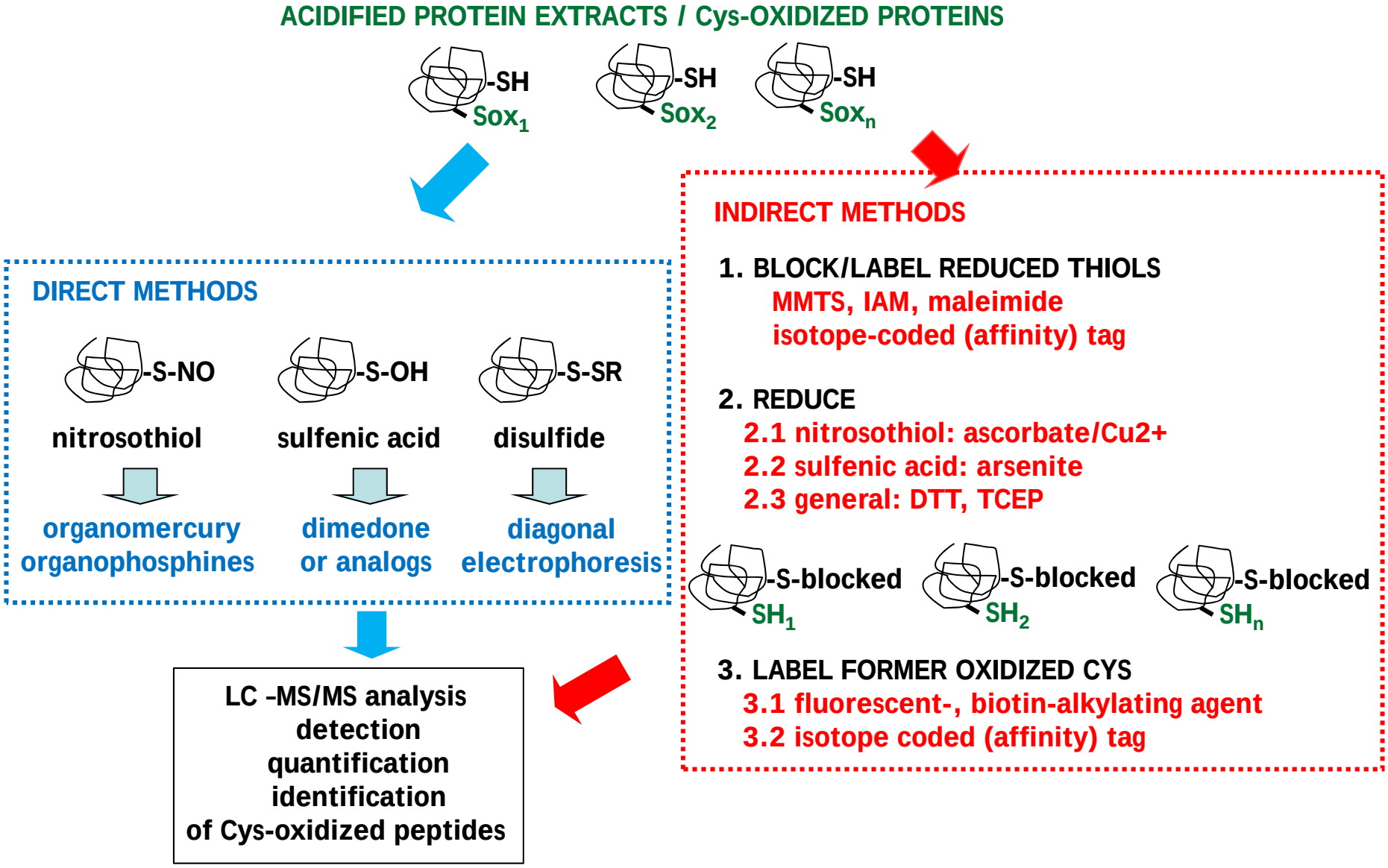
Figure 2. Summary of the different methods to study reversible Cys oxidation at the proteome level by MS. Oxidized Cys in proteins are studied by direct methods, shown in blue, or by indirect methods, shown in red. Direct methods: specific Cys modifications (S-NO and S-OH) are selected by specific reagents. Alternatively, disulfide-containing proteins are isolated after diagonal electrophoresis. In both cases, proteins are then identified by MS. Indirect methods are based on the labeling of oxidized Cys, shown in green, with alkylating agents, and require two previous steps: the blocking or labeling of reduced thiols in proteins and the reduction of originally oxidized Cys with specific or general reducing agents. See text for details.

Figure 3. Scheme representing the two chemical reactions used to derivatize carbonyls in proteins. (A) The reaction of hydrazides with carbonyls results in a hydrazone bonds that are further stabilized to amine bonds by chemical reduction with $NaCNBH_3$. (B) The reaction of aminooxy groups with carbonyls results in stable oxime bonds.

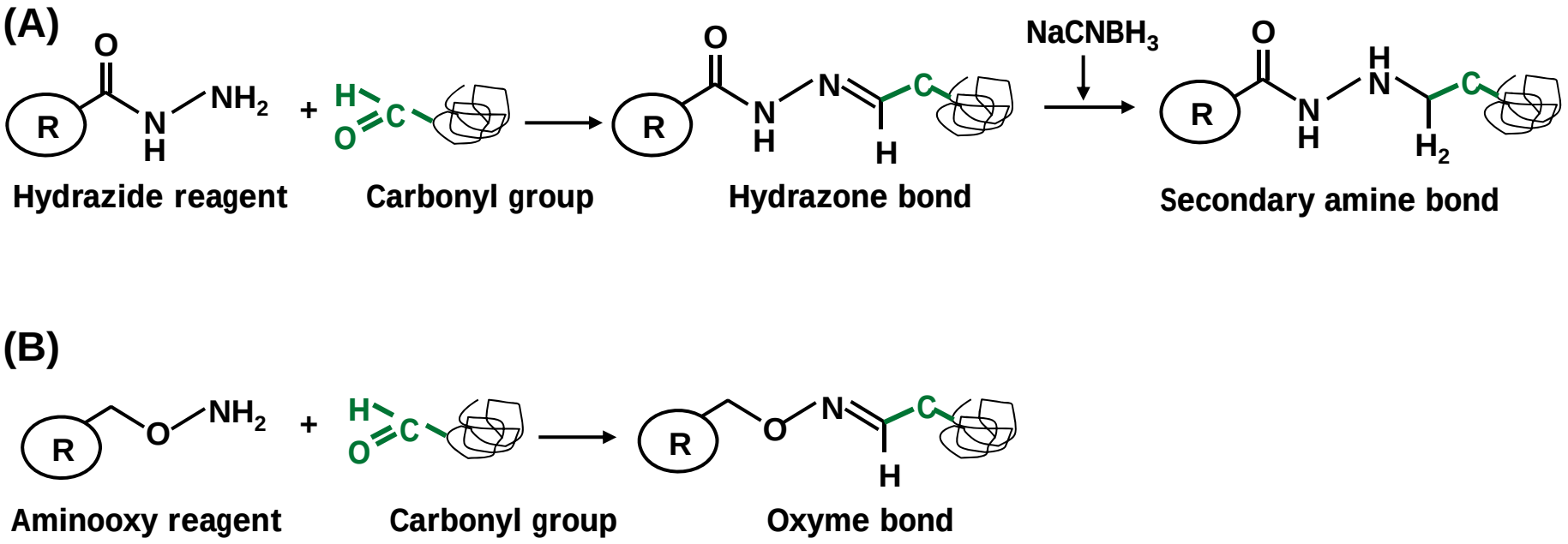
Figure 4. Summary of the different methods to study carbonylated proteins at the proteome level by MS. Carbonylated peptides can be detected by MS by reversible derivatization, shown in blue, or by irreversible tagging, shown in red. Reversible derivatization methods: the solid phase enrichment of carbonylated peptides is based on the reversible trapping of carbonylated peptides with hydrazides fixed to solid phases; peptides are then released for LC-MS. An optional step prior to enrichment (dashed square) is to use isotopic reagents specific for primary amines to quantify the total amount of a given peptide in different biological samples. Detection of irreversibly tagged carbonyls in peptides: carbonylated proteins are labeled with either non isotopic tags (left panels on red square), that rely on different chemistries towards carbonyl groups, or with isotopic/isobaric tags (right panels on red square); after labeling, peptides are enriched prior to detection, identification and relative quantification by LC-MS/MS.



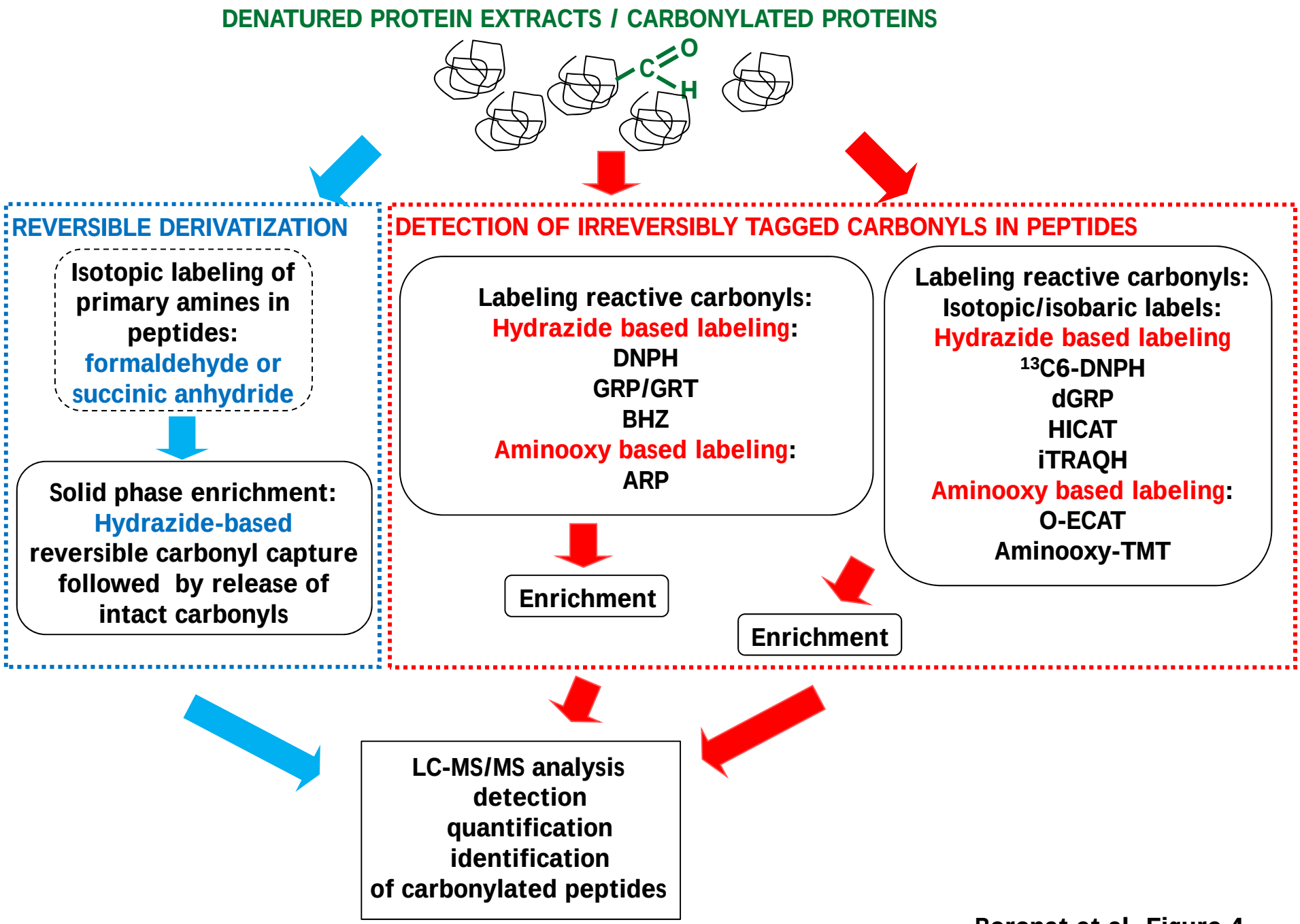
Boronat et al. Figure 1



Boronat et al. Figure 2



Boronat et al. Figure 3



Boronat et al. Figure 4